#8

It is therefore to be understood that within the scope of the appended claims, the invention may be practiced in a different way from what is specifically described herein.

All of the above cited disclosures of patents, publications and database entries are specifically incorporated herein by reference in their entirety to the same extent as if each such individual patent, publication or entry were specifically and individually indicated to be incorporated by reference.

Figures Legends

Figure 1 illustrates a first alternative to produce recombinant minimal vector using chimeric viruses. ψ represents the encapsidation region of the adenoviral constructs, H means human and B bovine origins.

Figure 2 illustrates a second alternative to produce recombinant minimal vector using chimeric viruses. ψ, H and B are as in Figure 1.

Figure 3 illustrates the basic set of Ad5 helper constructs. ψ, H and B are as in Figure 1.

Figure 4 illustrates Ad5 helper constructs which exploit complementation of a functional E1 region placed under the control of the endogenous E1 promoter (pTG 14927 and pTG 14929) supplied with either a small bovine encapsidation region (pTG 14927 0,33 kb) or a longer one (pTG 14929 0.84 kb). pTG13366 (small bovine encapsidation region) and pTG 13367 (long bovine encapsidation region) are control constructs without a functional E1 region.

Figure 5 illustrates Ad5 helper constructs which exploit complementation of a functional E1 region placed under the control of the murine PGK promoter. ITR represents BAV3 inverted terminal repeat sequences, ψ represents bovine encapsidation region (0.84 kb), ψ? represents BAV3 sequence potentially implicated in packaging (0.12kb) and PGK>E1 represents the Ad5 region driven by the murine PGK promoter.

Figure 6 illustrates the minimal vector constructs AdTG 14899 and AdTg 15202.

Figure 7 illustrates the adenoviral origin of replication. The binding sites for the viral pTP-pol complex and cellular factors NFI and Oct-1 are indicated for the Ad5 sequence (SEQ ID NO.:5). The sequence from BAV3 (SEQ ID NO.:6) is shown aligned with the sequence from Ad5.

generated by homologous recombination between a transfer plasmid named pTG 14866 (treated with XbaI and calf intestinal phosphatase, CIP) and a plasmid containing a modified version of the hFIX gene (pTG 14869, digested with SfiI). pTG 14866 contains the Ad5 cis-acting elements plus promoter sequences and the 3' end of the hFIX gene, separated by a unique XbaI site and was constructed as follows. pTG 8343, containing the 5' end of the Ad5 genome with a deletion in the E1 region is used as a source for the 5' end of the minimal vector. It is digested with AatII-BglII and overhanging end are made blunt using Klenow enzyme. The Ad5 3' ITR is isolated from plasmid pTG 5670 which contains the ITR, flanked by a PacI site on one end and a multiple cloning site on the other. The ITR is excised as a BglII fragment, treated with Klenow enzyme and ligated into pTG 8343 prepared as described above. The resulting plasmid, pTG 14138, contains the 5' end from Ad5 from nt 1 to 454, a MCS and the 3' ITR from nt 35828-35938. 5'flanking sequences from the hFIX gene are isolated from pTG 3960 (described in Jallat et al.) as a 1.4 kb fragment by digestion with PstI and HindIII, and introduced in PstI-HindIII digested pTG 14138 by ligation resulting in pTG 14159. Next, 3' flanking sequences from the hFIX gene are isolated from pTG 3960 as a PCR fragment. To this end, primers OTG 2070 (SEQ ID NO:1) (5'-AGAGCTTGTATGGTTATGGAGG-3') and OTG 12244 (SEQ ID NO:2) (5'-CACGATACTCGATGCAAGAC-3') are used to amplify a 1.6 kb fragment which is introduced by ligation in pTG 14159 which was linearized with XbaI, and made blunt using Klenow enzyme. This finally resulted in transfer plasmid pTG 14866. Before hFIX sequences were introduced in this transfer plasmid, we removed 5 consecutive PacI sites present in intron D of the hFIX gene in plasmid pTG 3960. This is necessary, because we will use PacI to excise the final minimal vector from the plasmid vector backbone. If PacI sites are present in the minimal vector sequences, this will result in fragmentation of the minimal vector genome. In order to

remove the PacI sites in pTG 3960, the plasmid was digested with PacI, treated with T4 DNA polymerase to remove 3' overhanging ends and re-circularized. The resulting plasmid is named pTG 14869. In addition to the deletion of the PacI sites, a PvuII fragment is deleted in intron A (4.8 kb) and there is a large deletion in intron F (7.1 kb). These modifications were already present in pTG 3960 and are described in Jallat et al.

pTG 14872: In parallel with pTG 14342, a minimal vector containing hFIX as stuffer DNA was constructed. Instead of using transfer plasmid 14866 containing the 5' and 3' flanking sequences of the hFIX gene, pTG 14868 was used. It contains part of intron A downstream of the adenoviral 5' ITR and encapsidation signal instead of sequences upstream of the hFIX promoter. The construction was started with pTG 14138, which contains the Ad5 cis-acting sequences separated by a MCS (see above) and which was linearized in the MCS by digestion with HindIII followed by treatment with Klenow. Into this vector was ligated a 1.1 kb ScaI fragment from pTG 3960 containing part of intron A from the hFIX gene. pTG 14160, with the insert in the sense orientation was thus obtained. Next a unique BamHI site in the remainder of the MCS between the Ad5 packaging signal and the intron A insert was destroyed, while at the same time a NotI site was introduced. This was done by digesting pTG 14160 with BamHI and ligating oligonucleotide OTG 3497 treated with polynucleotide kinase in the BamHI site. OTG 3497 (SEQ ID NO:3) (5'-GATCGCGGCCGC-3') is capable of forming a NotI site flanked by BamHI compatible cohesive ends by auto-hybridization. The resulting plasmid, pTG 14867, is used to introduce the 3' flanking region of the hFIX gene needed for homologous recombination in the next step. The 3' flanking sequence is isolated as an XbaI-KpnI fragment from pTG 14866 and ligated into XbaI-KpnI digested pTG 14867. The resulting transfer plasmid pTG 14868 is treated with XbaI and SfiI, which allows for the

introduction of hFIX sequences by homologous recombination. The hFIX sequences were isolated from pTG 14869, digested with PvuI and XhoI. Thus a minimal vector construct containing hFIX sequences but lacking the promoter sequences and the first exon of the hFIX gene was obtained. The construct was named pTG 14872.

pTG 14742: We have chosen to introduce expression cassettes in the unique PmeI site of the empty minimal vector pTG 14872. The PmeI site is located in intron D. In order to facilitate introduction of foreign cassettes, we have sub-cloned a fragment containing the PmeI site in a small vector, flanked by NotI sites. Introduction of the expression cassette in the PmeI site can be done in this vector. After excision of the expression cassette with flanking hFIX sequences by the action of NotI endonuclease, the cassette can be introduced by homologous recombination in the empty minimal vector which has been linearized with PmeI. The first step in the construction of the transfer plasmid consisted in introducing an EcoRI site flanked by NotI sites in phagemid pBluescript II SK^+ which was purchased from Stratagene. The phagemid was digested with PvuII and CIP, thus removing a 445 bp fragment containing the MCS flanked by bacteriophage T3 and T7 promoter sequences. The restriction enzyme recognition sites were introduced by ligation using a polynucleotide kinase treated, self-complementary oligonucleotide (OTG 12753, (SEQ ID NO:4) 5'-CAGGCGGCCGCGAATTCGCGGCCGCCTG-3'). The resulting plasmid is named pTG 15154 and can be used directly to introduce a hFIX fragment. This was done by cutting pTG 15154 with EcoRI and removing terminal 5' phosphate groups with CIP followed by ligation involving a 3.0 kb MfeI fragment from pTG 3960 containing the PmeI site. The resulting transfer plasmid pTG 14742 can be used directly for the introduction of foreign DNA sequences.